Pesq. Vet. Bras. 37(12):1416-1422, dezembro 2017

Molecular and serological detection of *Theileria equi*, *Babesia caballi* and *Anaplasma phagocytophilum* in horses and ticks in Maranhão, Brazil[1]

Rita de Maria Seabra Nogueira[2]*, Arannadia Barbosa Silva[3,4], Tayra Pereira Sato[3,5], Joicy Cortez de Sá[2], Ana Clara Gomes dos Santos[2], Edvaldo Franco Amorim Filho[2], Tássia Lopes do Vale[2] and Gilberto Salles Gazêta[3,4]

ABSTRACT.- Nogueira R.M.S., Silva A.B., Sato T.P., Sá J.C., Santos A.C.G., Amorim Filho E.F., Vale T.L. & Gazêta G.S. 2017. **Molecular and serological detection of *Theileria equi*, *Babesia caballi* and *Anaplasma phagocytophilum* in horses and ticks in Maranhão, Brazil.** *Pesquisa Veterinária Brasileira 37(12):1416-1422.* Departamento de Patologia, Universidade Estadual do Maranhão, São Luis, MA 65055-970, Brazil. E-mail: grita62@hotmail.com

Equine piroplasmosis is a tick-borne disease caused by the intraeytrhocytic protozoans *Babesia caballi* and *Theileria equi*. It has been reported as a main equine parasitic disease. In addition, *Anaplasma phagocytophilum*, the causative agent of granulocytic ehrlichiosis, causes a seasonal disease in horses. Both diseases, can be detrimental to animal health. In this sense, blood samples and ticks were collected from 97 horses raised in the microregion of Baixada Maranhense, Maranhão State, Brazil. Serum samples were subjected to Indirect Fluorescence Antibody Test (IFAT) and blood samples and ticks to Polymerase Chain Reaction (PCR) to evaluate the infection by *Theileria equi*, *Babesia caballi* and *Anaplasma phagocytophilum*. The overall seroprevalence was 38.14%, 18.55% and 11.34% for *T. equi*, *B. caballi* and *A. phagocytophilum*, respectively. The results of PCR from blood samples showed 13.40% and 3.09% positive samples to *T. equi* and B. *caballi,* respectively. A total of 170 tick specimens were collected and identified as *Dermacentor nitens*, *Amblyomma cajennense* sensu lato and *Rhipicephalus* (*Boophilus*) *microplus*. It was detected 2.35% (4/170) and 0.59% (1/170) positive tick samples by PCR for *T. equi* and *B. caballi*, respectively. All samples were negative to *A. phagocytophilum*. No statically difference (p>0.05) was observed when gender, age, use of ectoparasiticide and tick presence were analyzed. A BLASTn analysis of the sequenced samples indicated 97 to 100% similarity with *T. equi* 18S rRNA gene sequences in GenBank and 98 to 100% with *B. caballi*. Genetic analysis classified the obtained sequences as *T. equi* and *B. caballi* cluster, respectively. It can be concluded that these pathogens occur and are circulating in the studied area.

INDEX TERMS: Vector-borne disease, *Theileria equi*, *Babesia caballi, Anaplasma phagocytophilum,* horses, ticks, Brazil.

RESUMO.- [**Detecção molecular e serológica de *Theileria equi*, *Babesia caballi* e *Anaplasma phagocytophilum* em equinos e carrapatos no Maranhão.**] A piroplasmose equina é uma doença transmitida por carrapatos causada pelos protozoários intraeritrocitários *Babesia caballi* e *Theileria equi*. É relatada como uma doença parasitária comum em equinos. Além disso, *Anaplasma phagocytophilum*, o agente causal da ehrlichiose granulocítica, causa uma doença sazonal em equinos. Ambas as doenças, podem ser prejudiciais para a saúde animal. Nesse sentido, amostras de sangue e carrapatos foram coletadas de 97 cavalos criados na microrregião da Baixada Maranhense, estado do Maranhão, Brasil.

[1] Received on January 3, 2017.
Accepted for publication on May 30, 2017.

[2] Departamento de Patologia, Universidade Estadual do Maranhão (UEMA), Cidade Universitária Paulo IV, São Luis, MA 65055-970, Brazil. *Corresponding author: grita62@hotmail.com

[3] Laboratório de Referência Nacional em Vetores das Riquetsioses (LIRN), IOC/Fiocruz, Avenida Brasil 4365, Manguinhos, Rio de Janeiro, RJ 21040-900, Brazil.

[4] Programa de Pós-Graduação em Biodiversidade e Saúde, IOC/Fiocruz, Pavilhão Arthur Neiva, Avenida Brasil 4365, Manguinhos, Rio de Janeiro, RJ 21040-360.

[5] Programa de Pós-Graduação em Comportamento e Biologia Animal, Universidade Federal de Juiz de Fora (UFJF), Rua José Lourenço s/n, Martelos, Juiz de Fora, MG 36036-330, Brazil.

As amostras de soro foram submetidas ao Teste de Imunofluorescência Indireta (RIFI) e amostras de sangue e os carrapatos a Reação da Polimerase em Cadeia (PCR) para avaliar a infecção por *Theileria equi*, *Babesia caballi* e *Anaplasma phagocytophilum*. A prevalência foi de 38,14%, 18,55% e 11,34% para *T. equi*, *B. caballi* e *A. phagocytophilum*, respectivamente. Os resultados da PCR para as amostras de sangue demonstraram 13,40% e 3,09% de positividade para *T. equi* e *B. caballi*, respectivamente. Um total de 170 specimens de carrapatos foi coletado e foram identificados *Dermacentor nitens*, *Amblyomma cajennense sensu lato* and *Rhipicephalus* (*Boophilus*) *microplus*. Obteve-se 2,35% (4/170) e 0,59% (1/170) positivos por PCR para *T. equi* e *B. caballi*, respectivamente. Todas as amostras foram negativas para *A. phagocytophilum*. Não houve diferença estatística significativa (p>0.05) em relação ao sexo, idade, uso de ectoparasiticida e presença de carrapatos. A análise BLASTn das amostras sequenciadas para gene 18S rRNA indicaram 97 a 100% de similaridade com *T. equi* e 98-100% com *B. caballi* no GenBank. Análises genéticas classificaram as sequencias obtidas no mesmo clado que *T. equi* e *B. caballi,* respectivamente. Podemos concluir que estes patógenos estão circulando na área de estudo.

TERMOS DE INDEXACÃO: Doença transmitida por vetores, *Theileria equi*, *Babesia caballi*, *Anaplasma phagocytophilum*, cavalos, carrapatos, Brasil.

INTRODUCTION

Equine piroplasmosis is a tick-borne disease caused by the intraeytrhocytic protozoans *Babesia caballi* and *Theileria equi* (Mehlhorn & Scheinh 1988, De Waal 1992, De Waal & Van Heerden 1994, 2004). It has been reported as a main equine parasitic disease due to direct (loose of performance and mortality) and indirect damage (threat to horse industry) caused to animal health (Nogueira et al. 2005). It has great impact on international movement of equines for importation purpose or sports practices since positive or seropositive animals are not allowed to enter in countries free (Friedhoff et al. 1990, Knowles 1996). Once infected the animals remain carriers for long periods and act as source of infection to ticks (De Waal 1992, Laus et al. 2015). It is endemic in tropical and subtropical regions, and some temperate zones of the world (Shkap et al. 1998, Steinman et al. 2012). Mixed infections by *B. caballi* and *T. equi* are common in endemic areas (Scoles & Ueti 2015).

Anaplasma phagocytophilum, the causative agent of granulocytic ehrlichiosis, causes a seasonal disease. Members of the *A. phagocytophilum* complex have been recognized as worldwide tick-borne agents for several species of wild and domestic mammals. Besides, bacteria within this complex have recently emerged as zoonotic agents (Passamonti et al. 2010). In Brazil there are few reports about the infection as well as its natural vector.

The northeastern of Brazil has the second biggest horse herd. In the State of Maranhão, the microregion of Baixada Maranhense is characterized by an extensive group of lakes and lagoons with dry and rainy periods (Pinheiro et al. 2005). In this region calls the attention a native horse named "baixadeiro horse" that is used to work and therefore assumes a great local social economic importance.

In Maranhão State limited information are available regarding the infection by these three agents in horses; therefore the purpose of the present survey was to determine the relative frequency of *B. caballi*, *T. equi* and *A. phagocytophilum* by testing horses by Indirect Immunofluorescence Test (IFAT) and molecular methods (Polymerase Chain Reaction-PCR). Ticks were also analysed.

MATERIALS AND METHODS

Animals and sampling. The study was conducted from may/2012 to may/2013 in the microregion of Baixada Maranhense, in the municipalities of Santa Helena (02° 13' 51" S 45° 18' 00" O), Pinheiro (02° 31' 15" S 45° 04' 58" O) and Viana (03° 13' 12" S 45° 00' 14" O) in the Maranhão State northeastern Brazil. Ninety seven horses were randomly selected from three municipalities of Baixada Maranhense. Data from each horse including age and gender were recorded.

Blood samples (10mL) from each horse were collected from the jugular vein and placed into serum and EDTA tube. The serum tubes were centrifugated and sera were separated. Both sera and blood EDTA tubes were stored at -20°C until the time for serological and molecular analysis.

The horses were searched for ticks (approximately one minute per horse) which were removed manually and preserved in 70% ethanol for identification and PCR assays. Ticks were identified by the use of identification keys (Aragão & Fonsceca 1961, Barros-Battesti et al. 2006).

Indirect fluorescent antibody test (IFAT). Samples were screened for IgG antibodies against *Babesia caballi, Theileria equi* and *Anaplasma phagocytophilum* using a commercially available IFAT according to the manufacturer's instructions (Fuller Laboratories USA).

Initially samples were scored and it was considered positive sera that reacted at the dilution of 1:80. Seropositive samples were analyzed to determine the titration end point and antibodies titers were summarized in the following groups: 1:60, 1:160, 1:320, 1:640, 1:1280. Positive and negative serum controls, provided by the kit, were added to each slide.

Polymerase Chain Reaction (PCR) and sequencing. Genomic DNA was extracted from the blood samples by using QIAmp DNA Minikit (QIAGEN Hilden Germany) according to the manufacturer's instructions and stored at -20°C until use. Sampled ticks were submitted to DNA genomic extraction used guanidine isothiocyanate phenol technique, as described by Chomekzynski (1993) and modified by Sangioni et al. (2005).

For the detection of *B. caballi* and *T. equi* the extracted material was subjected to a PCR using primers previously described by Criado-Fornelio et al. (2003): BT-F1=5-GGTTGATCCTGCCAGTAGT-3, BT-R1=5-GCCTGCTGCCTTCCTTA-3 e BT-R2 = 5-TTGCGACCATACTCCCCCCA-3 that amplifies a region of 395bp for *Babesia* spp. and 410bp for *Theleiria* spp. of 18S rRNA gene.

For the detection of *A. phagocytophilum* DNA the extracted material was subjected to PCR primers MSP3F=5--CCAGCGTTTAGCAAGATAAGAG-3 and MSP3R=5-GCCCAGTAACAACATCATAAGC-3 that amplifies a fragment of 334bp of the *p44* gene (Zeidner et al. 2000).

The PCR products were detected by electrophoresis on 2% agarose gel (100mL TAE 0.5%, 2g agarose Ultra Pure™ Agarose Invitrogem™) stained with ethidium bromide distinct bands of *B. caballi T. equi* and *A. phagocytophilum* were visualized by UV transilluminator.

Positive products were selected purified and subjected for sequence confirmation in an automatic sequencer (ABI 3730xL - Applied Biosystems Foster City CA USA) according to Otto et al. (2008). The obtained sequences for each gene were analyzed and edited in ChromasPRO 1.5 (Technelysium Queensland Australia). Comparisons with sequences deposited in GenBank were done using BLASTn.

The sequences identified were aligned using software program ClustalW available in the MEGA 5.2. A phylogenetic tree was constructed with Neighbor-Joining at MEGA 5.2 program (Tamura el al. 2011) using the evolutive model Kimura-2-parameteres (Kimura 1980) with 1000 repetitions and exclusion of gaps.

Data analysis. Statistical associations of seropositivity to tick-borne pathogens with potential risk factors with the variables gender, age, use of ectoparasiticides and presence of ticks in the moment of the collection were performed. Data were tested by means of the chi-square or Fischer`s exact test, when necessary. The Odds Ratio (OR) was calculated for each variable with 95% confidence limits ($p<0.05$). All analyses were performed using the Epi Info software, version 6.04d (CDC, Atlanta, GA, USA).

RESULTS

Blood samples were obtained from 97 horses from Baixada Maranhense microregion. By means of IFAT, 37 (38.14%) samples reacted to *Theileria equi*, and 18 (18.55%) for *Babesia caballi* with titers of 1:80 to 1:1280 for both protozoans. 11 (11.34%) samples reacted to *Anaplasma phagocytophilum*, titers varying from 1:80 to 1:320 (Table 1).

There was no statistical difference ($p>0.05$) among the

Table 1. Frequency of IFAT IgG antibody titers against *Theileria equi*, *Babesia caballi* and *Anaplasma phagocytophilum* in naturally infected horses (n=97) raised in the microregion of Baixada Maranhense, Maranhão State, Brazil

	Titers of IgG antibodies					Seroreactive
	1:80	1:160	1:320	1:640	1:1280	animals
Anti-*T. equi*	9	4	4	10	10	37
Anti-*B. caballi*	4	2	2	8	2	18
Anti- *A. phagocytophilum*	2	6	3	-	-	11

Table 2. Distribution of the variables gender, age, use of ectoparasiticide and presence of ticks in horses seropositive for *Theileria equi* in the microregion of Baixada Maranhense, Maranhão State, Brazil

Variable		Reactive *T. equi*		Non reactive *T. equi*		Total	OR	IC	Value-P
		N	%	N	%				
Gender	M	9	26.47	25	73.53	34	0.45	0.16-1.22	0.1285628(a)
	F	28	44.44	35	55.56	63			
Age (years)	<1	2	15.38	11	84.62	13			
	1-4	5	18.52	22	81.48	27	0.80	0.09-6.00	0.5917915(b)
	5-8	17	53.12	15	46.88	32	0.16	0.02-0.98	0.0465580(a)*
	9- 2	10	62.50	6	37.50	16	0.11	0.01-0.84	0.0290414(a)*
	>13	3	33.33	6	66.67	9	0.36	0.03-3.96	0.3157895(b
Use of ectoparasiticide	Yes	20	37.74	33	62.26	53	0.96	0.39-2.38	0.9052469(a)
	No	17	38.64	27	61.36	44			
Presence of ticks	Yes	32	37.65	53	63.35	85	0.85	0.22-3.40	0.5116261(a)
	No	5	41.67	7	58.33	12			

M = male, F = female, (a) = Chi-Square Test, (b) = Fisher's Exact Test, OR = Odds Ratio, IC = Confidence Interval; * Significative difference.

Table 3. Distribution of the variables gender, age, use of ectoparasiticide and presence of ticks in horses seropositive for *Babesia caballi* in the microregion of Baixada Maranhense, Maranhão State, Brazil

Variable		Reactive *B. caballi*		Non reactive *B. caballi*		Total	OR	IC	Value-P
		N	%	N	%				
Gender	M	4	11.76	30	88.24	34	0.47	0.12-1.72	0.3219866(a)
	F	14	22.22	49	77.78	63			
Age (years)	<1	-	-	-	-	13			
	1-4	-	-	-	-	27			
	5-8	11	34.38	21	65.62	32			
	9-12	4	25	12	75	16	1.57	0.35-7.50	0.7411815(a)
	>13	3	33.34	6	66.66	9	1.05	0.18-6.63	0.6403036(b)
Use of ectoparasiticide	Yes	13	24.53	40	75.47	53	2.53	0.74-9.09	0.1620887(a)
	No	5	11.36	39	88.64	44			
Presence of ticks	Yes	16	18.82	69	81.18	85	1.16	0.20-8.51	0.6092381(b)
	No	2	16.67	10	83.33	12			

M = male, F = female, (a) = Chi-Square Test, (b) = Fisher's Exact Test, OR = Odds Ratio, IC = Confidence Interval.

Table 4. Distribution of the variables gender, age, use of ectoparasiticide and presence of ticks in horses seropositive for *Anaplasma phagocytophilum* in the microregion of Baixada Maranhense, Maranhão State, Brazil

Variable		Reactive *A. phagocytophilum*		Non reactive *A. phagocytophilum*		Total	OR	IC	Value-P
		N	%	N	%				
Gender	M	4	11.76	30	88.24	34	1.07	0.24-4.53	0.5836453(a)
	F	7	11.11	56	88.89	63			
Age (years)	< 1	3	23.07	10	76.93	13			
	1-4	2	7.40	25	92.6	27	3.75	0.41-39.15	0.1838549(a)
	5 a 8	3	9.37	29	90.63	32	2.90	0.38-22.67	0.2229695(a)
	9 a 12	2	12.50	14	87.50	16	2.10	0.22-22.83	0.3961686(a)
	>13	1	11.11	8	88.89	9	2.40	0.16-73.20	0.4496241(a)
Use of ectoparasiticide	Yes	6	11.32	47	88.68	53	1.00	0.24-4.14	0.6200802(a)
	No	5	11.36	39	88.64	44			
Presence of ticks	Yes	10	8.5	75	91.5	85	1.47	0.16-33.56	0.5919983(a)
	No	1	8.33	11	91.67	12			

M = male, F = female, (a) = Fisher's Exact Test, OR = Odds ratio, IC = Confidence interval.

studied variables (gender, age, use of ectoparasiticide and presence of ticks) in relation to *T. equi*, *B. caballi* and *A. phagocytophilum* infection (Table 2, 3 and 4). However horses aged from 5 to 8 years and 9 to 12 years ($p<0.05$) had more chance to get the infection by *T. equi* (Table 2).

The results of PCR and sequencing of DNA showed 13.40% (13/97) of horses were positive to *T. equi* and 3.09% (3/97) for *B. caballi*. All samples were negative to *A. phagocytophilum*.

A total of 170 ticks specimens were collected and the following species were identified: *Dermacentor nitens* (151), *Amblyomma cajennense* sensu lato (13) and *Rhipicephalus* (*Boophilus*) *microplus* (6). We obtained 2.35% (4/170) and 0.59% (1/170) positive by PCR for *T. equi* and *B. caballi*, respectively. All tick specimens were negative for *A. phagocytophilum*. The positive tick species for *T. equi* were *R.* (*B.*) *microplus* and *D. nitens*. Only *D. nitens* was positive to *B. caballi*.

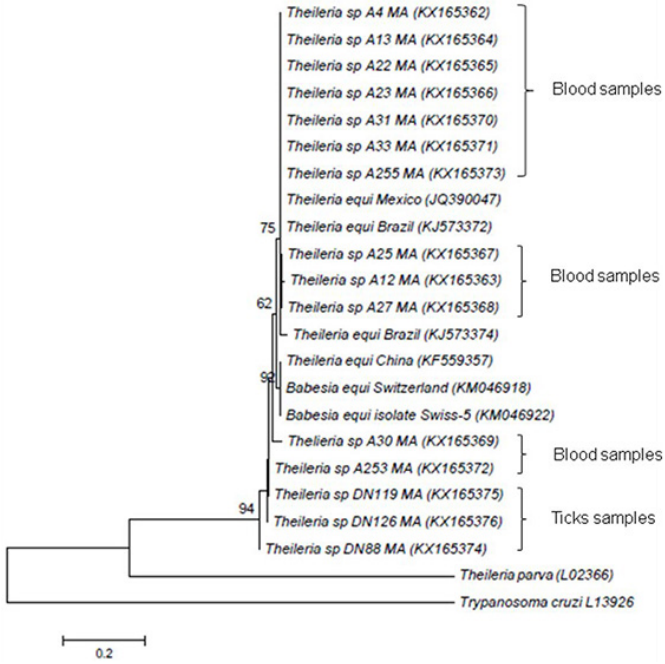

Fig.1. Phylogenetic tree of *Theileria* sp. using 18S rRNA gene sequences constructed by neighbor-joining method with Kimura two-parameter as evolution model and based on the nucleotide sequences. The GenBank accession codes are presented in parenthesis. The numbers at nodes are the bootstrap values obtained from 1,000 re-samplings. Bootstrap values below 60% are not present. DN = *Dermacentor nitens.*

Similarity analysis from the sequences obtained showed identity of 97% to 100% with *T. equi* (access GenBank JQ390047, KJ573372, KF559357). The phylogenetic dendogram formed by the sequences derivate from the products based on gene 18S rRNA demonstrated that the sequences obtained in this study are phylogenetically near *T. equi*. The 15 analyzed sequences formed a group along with *T. equi* (KJ573372, KJ573374, JQ390047 and KF559357) and *Babesia equi* (KM046918 and KM046922) (Fig.1). Partial sequence of the 18S rRNA gene from *Theileria* sp generated was deposited into GenBank with an accession number (KX165362-KX165376).

Similarity analysis from the sequences obtained showed identity 98% to 100% with *B. caballi* (access AB734392 and KJ549665). To analyze the sequences of gene 18S rRNA of *Babesia* spp. we noted that in the sequences of this study are phylogenetically near *B. caballi* and aligned forming a group with the sequences of *B. caballi* Brazil (KJ549665) and *B. caballi* Mongolia (AB734392) with bootstrap support 76%, as shown in Fig.2. Partial sequence of the 18S rRNA gene from *Babesia* sp. generated was deposited into GenBank with an accession number (KX165377- KX165380).

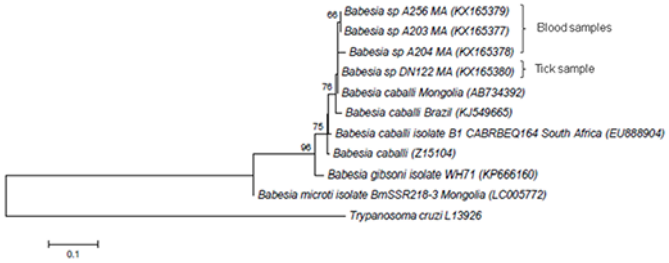

Fig.2. Phylogenetic tree of *Babesia* sp. using 18S rRNA gene sequences. Neighbor-joining analysis with 2-Kimura-parameters evolutionary model was performed. The GenBank accession codes are presented in parenthesis. The numbers at nodes are the bootstrap values obtained from 1,000 re-samplings. Bootstrap values bellow 60% are not present. DN = *Dermacentor nitens.*

DISCUSSION

Serological studies related to *Theileria equi* and *Babesia caballi* infections have demonstrated that these agents are widely distributed in Brazil with prevalence ranging from 7 to 90% (Heim et al. 2007, Kerber et al. 2009, García-Bocanegra et al. 2013). Furthermore, other researchers in Brazil pointed out that the main tick species infesting horses in Brazil are *Dermacentor nitens*, *Amblyomma cajennense* s.l. and *R.* (*B.*) *microplus*, same tick species identified in this work. In the American continent *D. nitens* is the only known vector of *B. caballi* (Roby & Anthony 1963) and in Brazil the prevalence of this protozoan coincides with the distribution of this tick species. Studies performed by Guerra & Brito (2004) reported a high prevalence of *D. nitens* in horses in Maranhão State.

This work detected *R. (B.) microplus* tick infected with *T. equi. R.* (*B.*) *microplus* which is a monoxenous tick and has cattle as it's the main host. However, horses may become alternative hosts in environments with there is contact between cattle and horses. It can be suggested that, at least, in Brazil, *R. (B.) microplus*, main tick in cattle and in many areas in equines, plays an important role in the transmission of *T. equi* (Torres et al. 2012). In addition, the vector competence of *R.* (*B.*) *microplus* has been demonstrated by the transtadial and intrastadial survival of *T. equi* in this tick specie (Ueti et al. 2008). Evidenciating the importance of this species of tick in the maintenance of the enzootic cycle of *T. equi.*

In the present study a greater seroprevalence was observed for *T. equi* then for *B. caballi*. Special attention should be given to the fact that a greater presence of *T. equi* was detected, because this agent is considered more pathogenic (Posada-Guzmán et al. 2015). These data differs from the one reported by Kerber et al. (2009) that studied the infection by both agents and the association with tick infestation in São Paulo State. They found out higher prevalence for *B. caballi*. Tick species *D. nitens*, *A. cajennense* and *R.* (*B.*) *microplus* were identified in 95%, 50% and 4% in horses, respectively. Moreover the infestations by *D. nitens* were more associated to *T. equi*.

Some factors can be associated to infection as proved by García-Bocanegra et al. (2013) in Spain. They reported different seroprevalence to *B. caballi* in mules (32.1%), donkeys (17%) and horses (7.9%). While for *T. equi* the results were much higher. The risk related to a higher seroprevalence to *T. equi* increases with age, presence of ticks and vaccination against other disease. On the other hand, the risk factors to *B. caballi* were animal species, presence of ticks and presence of shelters. Our results showed no differences among gender, age and presence of ticks. But as observed by García-Bocanegra et al. (2013) the risk of infection by *T. equi* increases with age, the same observed in the data presented here. According to Malekifard et al. (2014) the absence of differences in age and sex groups may be due to high number of tick in the area and continuous exposure of young and old horses to infected ticks.

The diagnoses of Equine Piroplasmosis can be done by direct and indirect methods; however, molecular assays appears as useful tools to identify the infection (Figueroa et al. 1993, Bashiruddin et al. 1999, Nicolaiewsky et al. 2001, Peckle et al 2013, Malekifard et al. 2014, Dória et al. 2016, Mahmoud et al. 2016). Therefore, sensitive and specific tests for Equine Piroplasmosis diagnosis are required to prevent introduction of causative agents into countries that are regarded free of the infection or disease (Mahmoud et al. 2016).The results presented here indicate that PCR is a sensitive assay and proves that the causative agents of Equine Piroplasmosis are circulating in the studied area. Studies performed in Brazil using PCR or IFAT have also detected both agents in horses, as the one reported in Minas Gerais (Heim et al. 2007), Rio Grande do Sul (Torres et al. 2012), Rio de Janeiro (Peckle et al. 2013) and São Paulo (Dória et al. 2016). Antibodies against *Anaplasma phagocytophilum* were detected in low levels however all blood and tick samples were negative by PCR. So the occurrence of this pathogen and its vector remains scarce. The finding of this pathogen is important since it represents a potential zoonotic risk. According to Van Andel et al. (1998) the capability of horses to produce anti-*A. phagocytophilum* antibodies that can be detected by commercial tests for up to six months enables this animal species to be used as sentinels in preventing human outbreaks.

Recently in Brazil, Rolim et al. (2015) investigated the rate of anti-*A. phagocytophilum* antibodies in horses from the Cavalry Squadron and Regiment of Mounted Police and found out 12% (11/91) animals with titer≥1:80. Animals aged from five to 14 years presented the highest rate of positive reactions but antibodies were detected in all ages. There was no statistical difference between males and females. These authors did not search for ticks although they stated that the circulation of the parasite among animals was not dependent of tick infestations. The data presented here did not show difference in gender and age among sampled animals as well as tick infestation.

Previously studies in Brazil reported 65% (Salvagni et al. 2010) and 3% (Parra et al. 2009) using serologic methods. However these authors obtained negative results when PCR was used for the detection of the pathogen as also observed in the present study and by Dória et al. (2016) when sports and traction horses were examined. Although Passamonti et al. (2010) detected the infection using serologic and molecular methods in horses. No bacterial infection was detected from ticks collected in the horses.

On the other hand, M'ghirbi et al. (2012) using serologic and molecular methods to detect *A. phagocytophilum* in horses and ticks in Tunisia found out 67% (40/60) positive by IFAT and 13% by PCR, with non-significant regional and gender differences, but a significant breed difference. 3 specimens of *Hyalomma marginatum*, which was the predominant tick species (130/154) were positive.

According to Passamonti et al. (2010) a positive serologic test against a PCR-negative result could possibly correspond to the dated past infection since *A. phagocytophilum* infection is characterized by limited and short-last bacteraemia. Based on this assumption it can be supposed that the horses of the present study had contact with the pathogen in the past.

CONCLUSIONS

The results pointed out the occurrence and circulation of *Babesia caballi*, *Theileria equi* and *Anaplasma phagocytophilum* in the Baixada Maranhense region.

This appears to be the first report of *Anaplasma phagocytophilum* in horses in Maranhão State.

Acknowledgments.- To Prof. Daniel Moura Aguiar, from Laboratório de Laboratório de Virologia e Rickettsioses do Hospital Veterinário, Universidade Federal de Mato Grosso (UFMT), for providing the positive control of *A. phagocytophilum.* The authors would like to thanks Plataforma Genômica-Sequenciamento de DNA-RPT01A, PDTIS/Fiocruz. This investigation was suported by Fundação de Amparo à Pesquisa e ao Desenvolvimento Científico e Tecnológico do Maranhão (FAPEMA). We also thank FAPEMA for the scholarship for Arannadia Barbosa Silva.

REFERENCES

Aragão H. & Fonseca F. 1961. Notas de Ixodologia. VIII lista e chave para os representantes da fauna ixodológica brasileira. Mem. Inst. Oswaldo Cruz 59:115-129.

Barros-Battesti D.M., Arzua M. & Bechara G.H. 2006. Carrapatos de Importância Médico-Veterinária da Região Neotropical: um guia ilustrado para identificação de espécies. VOX/ICTTD-3/Butantan, São Paulo, p.1-4.

Bashiruddin J.B., Camma C. & Rebêlo E. 1999. Molecular detection of *Babesia equi* and *Babesia caballi* in horse blood by PCR amplification of part of the 16S rRNA gene. Vet. Parasitol. 84:75-83.

Chomekzynski P.A. 1993. A reagent for the single-step simultaneous isolation of RNA, DNA and proteins from cell and tissue samples. BioTechniques 153:532-537.

Criado-Fornelio A., Martinez-Marcos A., Buling-Saraña A. & Barba-Carretero J.C. 2003. Presence of *Haemobartonella felis* and piroplasmids in cats from southern Europe: a molecular study. Vet. Microbiol. 93:307-317.

De Waal D.T. 1992. Equine piroplamosis: a review. Braz. Vet. J. 148:6-14.

De Waal D.T. & Van Heerden J. 1994. Equine babesiosis, p.293-304. In: Coetzer J., Thomson G. & Tustin R. (Eds), Infectious Diseases of Livestock with Special Reference to South Africa. Vol.1. Oxford University Press, Cape Town.

De Waal D.T. & Van Heeren J. 2004. Equine piroplasmosis, p.425-434. In: Coetzer J.A.W., Thomson G.R. & Tustin R.C. (Eds), Infectious Diseases of Livestock with Special Reference to Southern Africa. Vol.1. 2nd ed. Oxford University Press, Cape Town.

Dória R.G.S., Passarelli D., Chequer T.N., Reginato G.M., Hayasaka Y.B., Neto P.F., Grigoletto R. & Freitas S.H. 2016. Investigação clínica e comparação do esfregaço sanguíneo e PCR para diagnóstico de hemoparasitas em equinos de esporte e tração (carroceiros). Pesq. Vet. Bras. 36:724-730.

Figueroa J.V., Chieves L.P., Johnson G.S. & Buening G.M. 1993. Multiplex polymerase chain reaction based assay for the detection of *Babesia bigemina*, *Babesia bovis* and *Anaplasma marginale* DNA in bovine blood. Vet. Parasitol. 50:69-81.

Friedhoff K.T., Tenter A.M. & Muller I. 1990. Haemoparasites of equines: impact on international trade of horses. Rev. Sci. Tech. 9:1187-1194.

García-Bocanegra I., Arenas-Montes A., Hernández E., Adaszek L., Carbonero A., Almería S., Jaén-Téllez J.A., Gutiérrez-Palomino P. & Arenas A. 2013. Seroprevalence and risk factors associated with *Babesia caballi* and *Theileria equi* infection in equids. Vet. J. 195:172-178.

Guerra R.M.S.N. & Brito D.R.B. 2004. Ixodofauna de mamíferos domesticados da ilha de São Luis, MA. Entomol. Vect. 11:435-444.

Heim A., Passos L.M., Ribeiro M.F., Costa-Júnior L.M., Bastos C.V., Cabral D.D., Hirzmann J. & Pfister K. 2007. Detection and molecular characterization of *Babesia caballi* and *Theileria equi* isolates from endemic areas of Braz. Parasitol. Res. 102:63-68.

Kerber C.E., Labruna M.B., Ferreira F., De Waal D.T., Knowles D.P. & Gennari S.M. 2009. Prevalence of equine Piroplasmosis and its association with tick infestation in the State of São Paulo, Brazil. Revta Bras. Parasitol. Vet. 1:1-8.

Kimura M. 1980. A simple method for estimating evolutionary rates of base substitutions through comparative studies of nucleotide sequences. J. Mol. Evol. 16:111-120.

Knowles D.P. 1996. Control of *Babesia equi* parasitemia. Parasitol. Today 12:195-198.

Laus F., Spaterna A., Faillace V., Veronesi F., Ravagnan S., Beribé F., Cerquetella M., Maligrana M. & Tesei B. 2015. Clinical investigation on *Theleria equi* and *Babesia caballi* infections in Italian donkeys. BMC Vet. Res. 11:100-107.

Mahmoud M., El-Ezz N.T.A., Abdel-Shafy S., Nassar S.A., El Namaky A.H., Khalil W.K.B., Knowles D., Kappmeyer L., Silva M.G. & Suarez C.E. 2016. Assessment of *Theileria equi* and *Babesia caballi* infections in equine populations in Egypt by molecular serological and hematological approaches. Parasites Vectors 9:1-10.

Malekifard F., Tacassoli M., Yakhchali M. & Darvishzadeh R. 2014. Detection of *Theileria equi* and *Babesia caballi* using microscopic and molecular methods in suburb of Urmia, Iran. Vet. Res. Forum 5:129-133.

Mehlhorn H. & Schein E. 1998. Redescription of *Babesia equi* Laveran, 1901 as *Theileria equi* Mehlhorn, Schein 1998. Parasitol. Res. 84:467-475.

M'ghirbi Y., Yaïch H., Ghorbel A. & Bouattour A. 2012. *Anaplasma phagocytoplhilum* in horses and ticks in Tunisia. Parasites Vectors 180:2-7.

Nicolaiewsky T.B., Richter M.F., Lunge V.R., Cunha C.W., Delagostin O., Ikuta N., Fonseca A.S., Silva S.S. & Ozaki L.S. 2001. Detection of *Babesia equi* Laveran, 1901 by nested polymerase chain reaction. Vet. Parasitol. 101:9-21.

Nogueira C.E.W., Silva S.S., Nizoli Q., Ribas L.M. & Albuquerque L.P.A.N. 2005. Efeito quimioprofilático do dipropionato de imidocarb na prevenção da agudização de babesiose equina em cavalos portadores da infecção. Hora Vet. 146:14-17.

Otto T.D., Vasconcellos E.A., Gomes L.H.F., Moreira A.S., Degrave W.M., Mendonça-Lima L. & Alves-Ferreira M. 2008. ChromaPipe: a pipeline for analysis, quality control and management for a DNA sequencing facility. Genet. Mol. Res. 7:861-871.

Parra A.C. 2009. Investigação diagnóstica de doença concomitante babesiose anaplasmose em rebanho equino por PCR e elisa. Faculdade de Medicina Veterinária e Zootecnia, Universidade de São Paulo, São Paulo, SP. (Apud <www.teses.usp.br/teses/disponiveis/10/.../tde.../Andrea_Cristina_Parra_doutorado.pdf>*) Dez. 2014.*

Passamonti F., Veronesi F., Cappelli K., Capomaccio S., Coppola G., Marenzoni M.L., Piergili F.D., Verini A.S. & Coletti A. 2010. *Anaplasma phagocytophilum* in horses and ticks: a preliminary survey of Central Italy. Comp. Immunol. Microbiol. Infect. Dis. 33:73-83.

Peckle M., Pires M.S., Dos Santos T.M., Roier E.C.R., Da Silva C.B., Vilela J.A.R., Santos H.A. & Massard C.L. 2013. Molecular epidemiology of *Theileria equi* in horses and their association with possible tick vectors in the state of Rio de Janeiro, Brazil. Parasitol. Res. 112:2017-2025.

Pinheiro C.U.B., Dos Santos V.M. & Ferreira F.R.R. 2005. Usos de subsistância de espécies vegetais na região da baixada maranhense. Amazônia Ciênc. Desenv. 1:235-250.

Posada-Guzmán M.F., Dolz G., Romero-Zúniga J.J. & Jiménez A.E. 2015. Detection of *Babesia caballi* and *Theileria equi* in blood from equyines from four indigenous communities in Costa Rica. Vet. Med. Interna 2015:1-6.

Roby T.O. & Anthony D.W. 1963. Transmission of equine piroplasmosis by *Dermacentor nitens.* J. Am. Vet. Med. Assoc. 142: 768-769.

Rolim M.F., Oliveira F.C.R., Graça F.A.S. & Brasil F.C. 2015. Serological evidence of exposure to *Anaplasma phagocytophilum* in horses from the Rio de Janeiro state mounted police bred in the urban zone. Ciênc. Anim. Bras. 16:377-387.

Salvagni C.A., Dagnone A.S., Gomes T.S., Mota J.S., Andrade G.M., Baldani C.D. & Machado R.Z. 2010. Serologic evidence of equine granulocytic anaplasmosis in horses from central west Brazil. Revta Bras. Parasitol. Vet. 19:135-140.

Sangioni L.A., Horta M.C., Vianna M.C.B., Gennari S.M., Soares R.M., Galvão M.A.M., Schumaker T.T.S., Ferreira F., Vidotto O. & Labruna M.B. 2005. Rickettsial infection in animals and Brazilian spotted fever endemicity. Emerg. Infect. Dis. 11:265-270.

Scoles G.A. & Ueti M.V. 2015. Vector ecology of equine piroplasmosis. Annu. Rev. Entomol. 60:561-580.

Shkap V., Cohen I., Leibovitz B., Savitsky Pipano E., Avni G., Shofer S., Giger U., Kappmeyer L. & Knowles D. 1998. Seroprevalence of *Babesia equi* among horses in Israel using competitive ELISA and IFA assay. Vet. Parasitol. 76:251-259.

Steinman A., Zimmerman T., Klement E., Lensky I.M., Berlin D., Gottlieb Y. & Baneth G. 2012. Demographic and environmental risk factors for infection by *Theileria equi* in 590 horses in Israel. Vet. Parasitol. 187:55-562.

Tamura K., Peterson D., Peterson N., Stecher G., Nei M. & Kumar S. 2011. MEGA5: molecular evolutionary genetics analysis using maximum likelihood, evolutionary distance, and maximum parsimony methods. Mol. Biol. Evol. 28:2731-2739.

Torres A.J., Finger I.S., Farias N.A.R., Nizoli L.Q., Silva S.S. & Nogueira C.E.W. 2012. Aspectos epidemiológicos da Theileriose equina e sua relação com o carrapato *Rhipicephalus (Boophilus) microplus* em duas propriedades na região da campanha do Rio Grande do Sul, Brasil. Revta Ibero--Latinoam. Parasitol. 71:70-77.

Ueti W.M., Palmer G.H., Scoles G.A., Kappmeyer L.S. & Knowles P.D. 2008. Persistently infected horses are reservoirs for intrastadial tick-borne transmission of the apicomplexan parasite *Babesia equi*. Infect. Immun. 76:3525-3529.

Van Andel A.E., Magnarelli L.A., Heimer R. & Wilson M.L. 1998. Development and duration of antibody response against *Ehrlichia equi* in horses. J. Am. Vet. Med. Assoc. 15:1910-1914.

Zeidner N.S., Burkot T.R., Massung R., Nicholson W.L., Dolan M.C., Rutherford J.S., Biggerstaff B.J. & Maupin G.O. 2000. Transmission of the agent of human granulocytic ehrlichiosis by *Ixodes spinipalpis* ticks: evidence of an enzootic cycle of dual infection with *Borrelia burgdorferi* in northern Colorado. J. Infect. Dis. 182:616-619.